EFFECT OF ISCHEMIA-REPERFUSION INJURY ON SKELETAL MUSCLE IN ADULT MALE ALBINO RATS (LIGHT AND ELECTRON MICROSCOPIC STUDY)

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ABSTRACT

Background: Ischemia-reperfusion (IR) injury refers to tissue damage caused when blood returns to a tissue after a period of ischemia.

Aim of this study: Ischemia reperfusion injury is a serious problem. So, the present study was designed to evaluate the effect of ischemia-reperfusion (IR) injury on skeletal muscle structure in an animal model.

Materials and methods: Adult (12-14 weeks, 180-200 grams) male albino rats (n = 20) obtained from the breeding animal house, Faculty of Medicine, Zagazig University. These rats were randomly equally divided into two main groups: control group I and group II ischemia reperfusion (IR) group. The common iliac artery in group II was clamped by an incision over medial site of the right hind extremity. After 3h ischemia period, the clamp was removed. Rats sacrificed 2 weeks following reperfusion. At the time of sacrifice, all rats were anesthetized and blood samples were collected for various biochemical parameters. The gastrocnemius muscle was dissected out and processed for different histological analysis.

Results: These results showed that IR adversely affected skeletal muscle structure of the adult male albino rat.

Conclusion: These results confirm the necessity to develop ways to prevent muscle damage induced by IR injury and promote muscle regeneration.

Keywords: skeletal muscle, ischemia reperfusion injury, rat.

I. INTRODUCTION

Skeletal muscle is one of the most abundant tissues in the human body. It accounts for 40%–45% of the total body. It contains 50–75% of all body proteins. It is dynamically regulated by the balance between muscle protein synthesis and degradation [1]. It also has the ability to convert the chemical energy into mechanical energy therefore generating movement [2].

Moreover, it is a reserve of glucose and amino acids for different tissues such as skin, heart and brain that can support protein synthesis or energy production elsewhere in the body when other sources are depleted as in severe starvation or long-term protein energy malnutrition [2&3].

Ischemia-reperfusion (IR) injury refers to tissue injury caused when blood returns to a tissue after a period of ischemia [4]. Skeletal muscles are common target for ischemic reperfusion (IR) injury because they have large mass in addition to predominant anaerobic metabolism during prolonged muscular contraction [5].

Lower limb ischemia is a frequent clinical problem resulting from trauma, hemorrhage, vascular occlusion, hypercoagulable states and thromboembolic events [6].

There is a significant increase in mortality and morbidity rate in periods following IR injury of extremities due to compartment syndrome, rhabdomyolysis, renal failure, limb loss, systemic inflammatory syndrome and respiratory injuries [5, 7].
The pathogenesis of IR injury is complex. It involves an intrinsic intracellular injury process during the ischemic phase. In addition, an induced inflammatory response during the reperfusion phase [8].

Under physiological conditions, the primary cellular sources of ROS in striated muscles include mitochondria, xanthine oxidase (XO) and NADPH oxidase (NOX) [9].

The first line defence mechanism includes antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) that preserve the cells by scavenging the free oxygen radicals (ROS). They are responsible for maintaining ROS at normal levels. However, antioxidant defenses can be overwhelmed under specific pathological conditions resulting in cellular oxidative stress as during IR injury [9, 10].

Xanthine oxidase (XO) is a major contributor to ROS production [11]. As in the presence of XO and oxygen (O2), hypoxanthine can be converted to xanthine, which simultaneously produces superoxide anions O2•− [12]. Since XO and hypoxanthine are quickly accumulated during ischemia, this results in increased (O2•−) production [10].

During ischemia, O2 is the limited substrate for the previous reaction but can be available upon reperfusion [13]. This could justify greater ROS formation during reperfusion than during ischemia [10]. So, oxidative stress which precedes mitochondrial dysfunction arises during ischemia is enhanced after reperfusion [14].

So, the present study was designed to evaluate the effect of ischemia-reperfusion (IR) injury on skeletal muscle structure in an animal model

II. MATERIALS AND METHODS

Animals

Twenty adult male albino rats weighing 180-200 g were used in the current study. They were obtained from the Animal House, Faculty of Medicine, Zagazig University. Every five rats were housed in a separate cage and received tap water and fed ad libitum.

All procedures were done according to the recommendations of the experimental Animal committee.

Experimental design

All surgical procedures were performed under anesthesia with intramuscular ketamine hydrochloride in a dose of 50 mg/kg body weight [15]. Anesthesia was maintained by an additional half dose every 90 minutes. The rats were divided into two groups, ten rats in each group as follow:

Group I: (control group): rats of this group equally subdivided in to five rats received no treatment and five rats that underwent the same surgical steps as mentioned in the experimental model but without clamping of the artery. The animals of two subgroups were sacrificed with their corresponding ischemia-reperfusion.

Group II (IR group): Following sterilization of the right limb, transverse groin incision was made. The common iliac artery was clamped by an incision over medial site of the right hind extremity. In addition, a rubber arterial tourniquet was applied at the level of the lesser trochanter to block collateral blood flow [16]. After 3h ischemia period, the clamp and tourniquet was removed. Rats sacrificed 2 weeks following reperfusion [17].

Ischemia and reperfusion of the limbs were confirmed by observation of changes in the color of the sole of the foot and by palpation the femoral artery.

At the end of the experiment for each group, blood samples were drawn from the femoral vein using heparinized syringes to measure the serum levels of lactate dehydrogenase enzyme (LDH) and creatine phosphokinase enzyme (CPK). The rats were sacrificed by intramuscular injection of ketamine hydrochloride (90 mg/kg)/xylazine (15 mg/kg). Muscle samples were taken from the gastrocnemius muscles.

General observations:

During the experimental period, the general behavior and gait of rats were checked daily.

Biochemical analysis:
Skeletal muscle injury was evaluated by measuring serum levels of released cytoplasmic enzymes lactate dehydrogenase enzyme (LDH) (Rat Lactate Dehydrogenase (LDH) ELISA Kit; MyBioSource, San Diego USA) and total creatine phosphokinase enzyme (CPK) release (Rat Creatine Kinase (CK) Enzymatic Assay Kit; Bio diagnostic, Egypt). The blood samples were centrifuged (3000 rotation /min for 15 min at 4 °C) to obtain plasma. Results were expressed in Units/L.

**Histological study:**

**Light microscopic study:**

The muscle samples of all groups were fixed in 10% formol solution and kept for 24 hours then dehydrated in ascending grades of alcohol and cleared in xylene then embedded into paraffin wax. Serial sections (5 µm thickness) were prepared for H&E and Mallory trichrome staining.

**Electron microscopic study:**

Small pieces of the muscles were fixed in 3% glutaraldhyde (PH 7.4) in phosphate buffer for 24 hours and post fixed in 2% osmium tetroxide in phosphate buffer for one hour. Ultrathin sections (80-90 nm) were stained with uranyl acetate and lead citrate. Tissue sections were evaluated using a JEOL transmission electron microscope JEM-1200. Ex, Japan.

**Morphometrical & statistical analysis**

Statistical analysis for morphological measurement, image analysis was done for H&E stained sections of the right gastrocnemius were used. They were assessed by an ordinary light microscope using Leica 500 image analyzer computer system (England) at the Image Analyzing Unit of the Pathology Department, Faculty of Dentistry, Cairo University. Statistical significant difference was determined by one-way analysis of variance (ANOVA), followed by LSD comparison between different groups. Statistical analysis is used for the biochemical results and the area% of collagen fibers.

### III. RESULTS

**General observations:**

During the course of the experiment, none of the rats died during the observation period or showed signs of gangrene or limb loss. Rats of the control group showed normal gait while rats of the IR group seemed to favor the non-ischemic leg during the early postoperative days.

**Biochemical results**

Table 1: Statistical analysis of the mean values of serum level of LDH and CK (IU/L) in different studied groups:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SD Serum LDH</th>
<th>Mean ± SD Serum CK</th>
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<tbody>
<tr>
<td>Group I (Control)</td>
<td>517.9±60.7</td>
<td>248.6±12.607</td>
</tr>
<tr>
<td>Group II (IR)</td>
<td>949.699±63.116**a</td>
<td>535.2±35.010**a</td>
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** P< 0.001 Highly statistically significant difference  **a value compared with control group

Non- significant difference was detected between the results of subgroups 1a, and 1b of the control group (p > 0.05). Thus, the average of group I was used as a control, to be compared with the other groups of the study. Statistical analysis revealed a highly significant increase in the mean serum LDH and CK in the ischemia reperfusion group as compared to the control group. (Table 1).
Histological results:

Light microscopic results:

Examination of the H&E-stained sections of the control gastrocnemius muscle (Figure 1A) showed normal skeletal muscle architecture. Little endomysial spaces were seen separating the skeletal muscle fibers. Skeletal muscle fibers were cylindrical, elongated and non-branched. Each muscle fiber had acidophilic cytoplasm with apparent transverse striations and numerous peripheral flat nuclei. The gastrocnemius muscle of ischemia-reperfusion injury group (Figure 1B) revealed degenerative changes in the skeletal muscle fibers including disruption of the skeletal muscle fibers with loss of striation, separation of the muscle fibers with increased endomysial spaces. Also, wide separation of the skeletal bundles was observed. Some fibers showed atrophic changes in the form of splitting of the muscle fibers and nuclear clumps. Fragmented and hyalinization of muscle fibers were observed (Figure 1C). Fat cells were detected between muscle fibers and congested blood vessel (Figure 1D).

![Figure 1. H&E-stained sections in the gastrocnemius muscle of all studied groups. Control group (1A): shows cylindrical elongated non-branched muscle fibers (F). The muscle fibers have acidophilic cytoplasm with transverse striations (arrow head) and numerous flattened peripheral nuclei (arrow). IR group(1B-D): 1B the gastrocnemius muscle showing marked disruption of the skeletal muscle fibers with loss of striation, separation of the skeletal muscle fibers with increased endomysial spaces (curved arrow) and wide separation of the skeletal bundles (*) with nuclear clumps in the sarcoplasm of some muscle fibers (arrow). 1C showing fragmented muscle fibers (F) and hyalinization of the sarcoplasm (double arrow). 1D showing fat cells (arrow) and congested blood vessels (V) among disarranged muscle fibers (F). [H&E, X400]](image)

Mallory trichrome stained sections of the control group revealed few collagen fibers in the perimysium (Figure 2A). While, Abundant collagen fibers were detected in the perimysium of the IR group (Figure 2B).
Figure 2. Mallory’s Trichrome-stained sections in the gastrocnemius muscle of all studied groups. The control group (2A) showing few collagen fibers (arrow) in the perimysium. The IR group (2B) shows abundant collagen fibers (arrow) in the perimysium [Mallory’s Trichrome X400].

Electron microscopic results

Ultrastructurally, the control group showed the normal arrangement of skeletal muscle fibers. Mitochondria were located in the inter-myofibrillar space and closer to the sarcolemma with blood vessel running between the muscle fibers (Figure 3A). In the IR group showed loss of the normal architecture of the muscular tissue in the form of fragmented myofibrils, cytoplasmic lysis, increased inter-myofibrillar spaces and interrupted Z lines. The sarcoplasm contained irregular shaped swollen mitochondria and nuclei with irregular outlines (Figure 3B). Other sections showed parts of myofibrils replaced by fibrous tissue (Figure 3C). Congested blood vessels and cellular infiltration were observed (Figure 3D).

Figure 3. Electron micrographs of the gastrocnemius muscle of all studied groups. Control group (3A) gastrocnemius muscle showing the regular arrangement of myofibrils and the mitochondria (arrow) in the sarcoplasm. IR group (3B-D): (3B) showing fragmentation of the myofibrils (wavy arrow) with increased inter-myofibrillar space (*) and interrupted Z line (arrow head). Some irregular shape swollen mitochondria (arrow) and nucleus (N) with irregular outlines were observed. (3C) showing muscle fibers partially replaced by fibrous tissue (arrow). (3D) showing irregularly arranged myofibrillar elements (F), congested blood vessel (V) surrounded by large amount of collagen fibers (double arrow) and cellular infiltrations around blood vessels (I) are also observed [Orig. Mag. X 1200]

Morphometrical and statistical results :The average of group I was used as a control, to be compared with the other groups of the study. There was highly significant increase in the area % of collagen fibers in the IR group when compared to the control group (Table 2).

Table 2: The mean area % for collagen fibers between studied groups
IV. DISCUSSION

Ischemia–reperfusion injury consists of two phases; early-phase injury due to xanthine oxidase or mitochondria-derived ROS (within several hours after reperfusion) and late-phase injury due to secondary production of ROS by infiltrating inflammatory cells [12].

In the current work, during the course of the experiment, rats of the recovery group were observed to favor the non-ischemic leg during the early postoperative days compared to the control rats that showed normal gait. Zhang et al. [18] clarified that impaired fatigue resistance and contraction were also observed after IR in gastrocnemius. Also, Rybalko et al. [19] demonstrated that increased deposition of connective tissue has a negative impact on contractile muscle function by decreasing myofiber occupancy.

Regarding LDH and CK serum levels, comparison in-between groups showed a highly statistically significant increase in the mean serum LDH and CK in the skeletal muscle of ischemia reperfusion group as compared to the control group.

Geng et al. [20] reported that when muscle ischemia reperfusion occurs, the release of inflammatory mediators is increased with large numbers of free radicals are generated. Hence, the permeability of the cell membrane is increased resulting in an increased levels of serum LDH and CK.

In the current work, examination of H&E stained longitudinal sections in the Rt gastrocnemius muscle of the skeletal muscle IR injury group revealed separation of the muscle fibers, fragmentated and hyalinized muscle fibers with increased endomysial spaces and nuclear clumps. Light microscopic results were confirmed by EM as it showed loss of the normal architecture of the muscular tissue in the form of fragmented myofibrils, cytoplasmic lysis, increased inter-myofibrillar spaces and interrupted Z lines. The sarcoplasm contained irregular shaped swollen mitochondria and nuclei with irregular outlines.

These results were in accordance with Nada et al. [21] & Kuroda et al. [22], they reported that muscle destruction including edema of cells, necrosis and destruction of muscle structure occurred after IR injury of skeletal muscle.

These degenerative changes could be explained as during ischemia, anaerobic metabolism due to accumulation of (lactate, glycerol and pyruvate) produces a decrease in cell pH. To buffer this accumulation of hydrogen ions, the Na+/H+ exchanger excretes excess hydrogen ions which produces a large influx of sodium ions and swelling of cells [23].

Low ATP concentrations which inactivates ATPases leads to dysfunction in ionic exchangers (Na+/K+-ATPases and Ca2+-ATPases), reversal of Na+/Ca2+ antiporter mechanism and limits the reuptake of calcium by the endoplasmic reticulum (ER). Thereby resulting in an accumulation of cytosolic Ca2+. These elevated intracellular Ca2+ levels are responsible for irreversible damage to cell integrity due to the activation of cellular degradation enzymes such as lysozymes and phospholipases [10].

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SD area% of collagen fibers</th>
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<tr>
<td>Group I (Control)</td>
<td>3.917±.63524</td>
</tr>
<tr>
<td>Group II (IR)</td>
<td>37.7800±2.704**</td>
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Anaerobic metabolism during IR resulting in decreases in pH, followed by reduction of available ATP and calcium overload in cells. This is accompanied by opening of the mitochondrial permeability transition pore (mPTP), disrupting mitochondrial membrane potential and further impairs ATP production [24].

Acidic cellular pH and NADH accumulation keep transient opening of the mPTP during ischemia which may serve to protect cells against cytosolic Ca2+ overload but during reperfusion the rapid resumption of oxidative phosphorylation and restoration of normal cytosolic pH results in mitochondrial Ca2+ uptake which opens the mPTP resulting in swelling of mitochondria with free radical release and cell death [25, 26].

Free radicals are highly reactive and unstable compounds that easily react with all cellular components (lipids, proteins, carbohydrates and nucleic acids) in the body inducing lipid and protein peroxidation and damage of nucleic acids leading to cell damage or cell death [27].

These events cause changes in transcapillary permeability, which leads to increased interstitial fluid pressure and capillary compression. As a result, cells undergo necrosis due to deficiency of metabolic nutrients and the direct attack by cellular infiltration [10].

Also, in the present study a highly statistically significant increase in the mean area % for collagen fibers in the IR group as compared to the control group.

This finding was in agreement with Vignaud et al. [28] who stated that fibrosis detected by sirius red stainings was observed in ischemic/reperfused muscles after 14 day indicating defective muscle regeneration.

Like most forms of tissue injury, ischemia excites an acute inflammatory response that could lead to infarction and tissue death followed by replacement of necrotic tissue by fibrous tissue, resulting in scarring. Increased endomysium and perimysium was clearly noticed as fibrosis is part of the response to muscle injury and incomplete recovery [17].

Fibrosis is defined as formation of a connective tissue scar due to prior activation of fibroblasts and fibro-adipogenic progenitors during inflammatory phase of IR [29, 30].

Also, fibroadipogenic progenitors proliferate following injury and differentiate into myofibroblasts, which are responsible for ECM deposition and fibrotic deposition [30].

In conclusion, the results of present study showed that ischemia reperfusion injury may have deleterious effects on the histological structure of the skeletal muscle that may affect function of skeletal muscle.

REFERENCES:


